



AA

# 4  
02CO

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to:

Assistant Commissioner of Patents

Washington, D.C. 20231 on September 17, 2001.

Patent Application

Docket No. GJE-53X

Serial No. 09/928,639

David Saliwanchik

David R. Saliwanchik, Patent Attorney

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit : not yet assigned  
Applicant(s) : Mikhail Sergeevich Shchepinov  
Serial No. : 09/928,639  
Filed : August 13, 2001  
For : Mass Spectrometry

Assistant Commissioner of Patents  
Washington, D.C. 20231

CLAIM OF PRIORITY UNDER 35 U.S.C §119

Sir:

The applicants hereby reaffirm their claim to the right of priority granted pursuant to 35 U.S.C. §119 based upon Great Britain applications Serial No. 0019994.3 (filed November 13, 2000).

A Certified copy of the above Great Britain application is being submitted herewith.

Respectfully submitted,

David Saliwanchik

David R. Saliwanchik

Patent Attorney

Registration No. 31,794

Phone No.: 352-375-8100

Fax No.: 352-372-5800

Address : 2421 N.W. 41st Street  
Suite A-1  
Gainesville, FL 32606

DRS/la

Attachments: Certified copy of GB application no. 0019994.3

1000

1000

1000

1000



INVESTOR IN PEOPLE

The Patent Office  
Concept House  
Cardiff Road  
Newport  
South Wales  
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

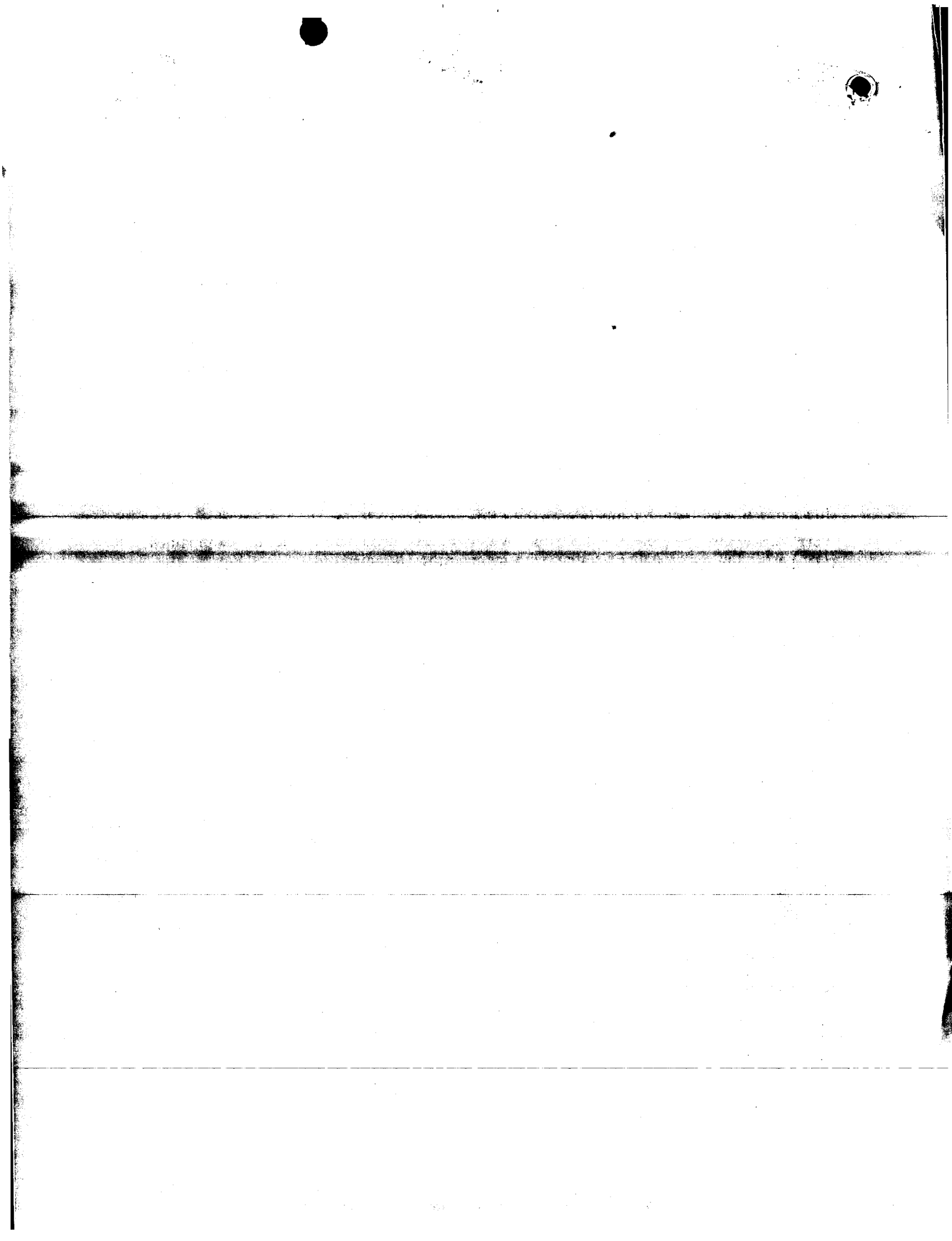
In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

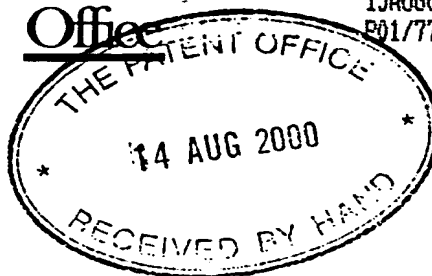
Signed

Dated

14 August 2001



The  
Patent  
Office



15AUG00 E560772-5 D02890  
P01/7700 0.00-0019994.3

The Patent Office

Cardiff Road  
Newport  
South Wales  
NP9 1RH

## Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

1. Your reference	LAS01192GB		
2. Patent application number (The Patent Office will fill in this part)	14 AUG 2000	0019994.3	
3. Full name, address and postcode of the or of each applicant (underline all surnames)	Isis Innovation Limited University Offices Wellington Square Oxford OX1 2JD		
Patents ADP number (if you know it)			
If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom		
4. Title of the invention	Mass Spectrometry		
5. Name of your agent (if you have one)	Gill Jennings & Every		
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	Broadgate House 7 Eldon Street London EC2M 7LH		
Patents ADP number (if you know it)	745002	✓	
6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number	Country	Priority application number (if you know it)	Date of filing (day / month / year)
7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application		Date of filing (day / month / year)
8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if: a) any applicant named in part 3 is not an inventor, or b) there is an inventor who is not named as an applicant, or c) any named applicant is a corporate body. See note (d))	YES		

# Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form

Description

7

Claim(s)

4

Abstract

Drawing(s)

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

NO

11. For the applicant  
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date

14 August 2000

12. Name and daytime telephone number of person to contact in the United Kingdom

PERRY, Robert Edward

020 7377 1377

## Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

## Notes

- If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.
- Write your answers in capital letters using black ink or you may type them.
- If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- Once you have filled in the form you must remember to sign and date it.
- For details of the fee and ways to pay please contact the Patent Office.

Mass Spectrometry

5 This invention relates to processes of measuring molecular mass by mass spectrometry, in particular to methods of precise calibration of mass spectrometers, and to kits and systems for use in calibrating mass spectrometers.

10 In recent years the required precision for measurements obtained by mass spectrometry has increased greatly. It is now routine that mass spectrometric data should have error not more than 1 to 5 ppm. Thus it has become increasingly important to obtain accurate calibration of mass spectrometers.

15 It is known to calibrate mass spectrometers by including calibration compounds of known molecular mass in the sample to be analysed. An ideal set of calibration compounds would include at least two compounds of different molecular mass, one above, one below and both close to the  
20 expected molecular mass of the material whose exact mass is to be measured. It is important that compounds of known mass have mass close to the mass to be measured, since the calibration curve is not linear.

25 Various types of compound are known for use as calibration compounds. These include certain sugar derivatives which can be provided with a range of molecular masses. However, they are difficult to synthesise and tend to break down during the mass spectrometry process into by-products which result in significant background noise in  
30 the spectrum. They tend also not to be particularly good flyers in the mass spectrometer.

Alternative systems are based on peptides. However these are again expensive to make. Additionally they tend to have different signal intensities and can be difficult  
35 to find in the final spectrum.

It would be desirable to provide methods and systems for calibration of mass spectrometers which include

calibration compounds of accurate and predetermined molecular mass, which fly well in the mass spectrometer and which do not give rise to breakdown products which produce background noise in the spectrum. It would also be desirable to provide calibration compounds which are straightforward and inexpensive to make.

According to the invention we provide a method of measuring the molecular mass of a compound Y of unknown molecular mass by mass spectrometry, comprising

10 providing a sample of compound Y,

providing samples of at least two different compounds each of formula (I), R-X in which R is a trityl group and X is cleavable to form a charged species for mass spectrometry,

15 and recording the molecular mass of compound Y and the at least two compounds of formula (I) in a mass spectrometer.

Thus in the invention we use the trityl compounds of formula (I) as calibration compounds in mass spectrometry. We find that the trityl groups are particularly suitable for use as calibration compounds. The trityl groups are readily cleaved from the compound of formula (I) by illumination with a laser in the mass spectrometer. Further, the resulting positively charged carbonium ion is very stable and thus sensitivity of detection is high. They also fly well in the mass spectrometer. Furthermore, the unique structure of the trityl group presents scope for a wide range of predetermined molecular masses which can be selected and controlled as appropriate depending upon the estimated molecular mass of the compound to be measured. Generally in the process the compound Y is not also of the formula R-X.

35 The calibration compounds are of formula R-X in which R is a trityl group. Generally R is  $R^1R^2R^3C-$  wherein  $R^1$ ,  $R^2$  and  $R^3$  are the same or different and each is a monocyclic or fused ring aromatic group that is substituted or unsubstituted. Preferably each of  $R^1$ ,  $R^2$  and  $R^3$  is aryl, preferably phenyl.



Preferably at least one of  $R^1$ ,  $R^2$  and  $R^3$  carries a substituent selected from  $C_1$ - $C_{20}$  alkoxy or hydrocarbyl, substituted or unsubstituted. Substituents may be present at any point in the aromatic ring, but *para* substituents are convenient and preferred.

When the alkoxy or hydrocarbyl is substituted the substituent is preferably selected from the group consisting of carboxylic acid, sulphonic acid, nitro, cyano, hydroxyl, thiol, primary, secondary or tertiary amino, primary or secondary amido, anhydride, carbonyl halide and active ester. In these substituents, hydrogen atoms may be partly or wholly replaced by deuterium or halogen, e.g., fluorine. This improves the range of molecular weights available. For instance, alteration of the molecular mass may be obtained by the use of non-radioactive isotopic substituents, e.g., small alkyl groups containing 1, 2 or 3 deuterium atoms.

It is preferred that the trityl group R is substituted with amide substituents. Substituted trityl groups of this type are particularly easy to produce, with a wide range of molecular masses. Thus preferably the trityl group R includes two or four or more amide substituents. More preferably  $R^1$ ,  $R^2$  and  $R^3$  together carry at least two amide groups and/or at least two reactive groups for coupling, preferably N-hydroxy hydroxysuccinimide ester groups.

The group X is cleavable to form a charged species for mass spectrometry. Usually it is photocleavable, e.g., by laser illumination. X may be any suitable leaving group, for instance halide or tosylate.

In the method at least two different compounds of formula (I), having different molecular mass, are used. Preferably the number of compounds of formula (I) is larger, for instance at least 5, preferably at least 10 and even at least 20 may be desirable. The compounds of formula (I) are generally selected to have a range of molecular masses around the estimated molecular weight of compound Y. Desirably the molecular masses of the

compounds of formula (I) are close to that of compound Y, for more accurate calibration. Thus in a preferred method according to the invention the molecular mass of unknown compound Y is estimated as  $M_y$  and at least one compound of formula (I) having known molecular mass  $M_1$  below  $M_y$  is provided, as is at least one different compound of formula (I) having molecular mass  $M_2$  above  $M_y$ . Preferably each of  $M_1$  and  $M_2$  lies in a molecular mass range not more than  $\pm 50\%$  of  $M_y$ . That is  $M_1$  is not less than 50% of  $M_y$  and  $M_2$  is not greater than 150% of  $M_y$ . Preferably the range is within  $\pm 25\%$ , more preferably  $\pm 10\%$ .

It is possible to provide prepared mixtures of at least two compounds of formula (I) which can be combined with a sample Y of unknown molecular mass and used as calibration compounds in mass spectrometry. Such mixtures preferably comprise at least 5, more preferably at least 10 and in some cases at least 20 different compounds of formula (I).

Thus in a second aspect of the invention we provide a method of measuring the molecular mass of a compound Y of unknown molecular mass comprising estimating the expected molecular mass of compound Y, selecting at least one calibration compound of formula (I) R-X having molecular mass close to the expected molecular mass of the compound Y, in which R is a trityl group and X is cleavable to form a charged species for mass spectrometry, and subjecting both compounds to mass spectrometry simultaneously.

In this aspect the compound of formula (I) may have any of the preferred features indicated for the first aspect of the invention above, as appropriate.

According to a third aspect of the invention we provide mixtures suitable for use in mass spectrometry as calibration compounds. According to this aspect we provide a set of calibration compounds for mass spectrometry comprising at least two separately packaged mixtures (a) and (b), wherein

mixture (a) comprises at least two different compounds each of formula (I) R-X and having different molecular masses

5 mixture (b) comprises at least two further compounds of formula (I) R-X having different molecular masses and wherein R is a trityl group and X is cleavable to form a charged species for mass spectrometry.

10 Thus the set comprises at least two different sets of calibration compounds. Preferably each set has molecular masses extending across a different range. The ranges may overlap. Thus preferably the lowest molecular mass in mixture (a) is lower than the lowest molecular mass in mixture (b) and the highest molecular mass in mixture (a) is lower than the highest molecular mass in mixture (b).

15 More preferably, the set comprises at least three separately packaged mixtures of compounds, more preferably at least five separately packaged mixtures of compounds. The greater the number of separate mixtures, the greater the scope for calibration of unknown compounds of a range of molecular masses.

Each set preferably contains at least five compounds of different molecular masses, more preferably at least ten different compounds of different molecular masses. In some cases at least twenty different compounds are desirable.

25 An advantage of the use of the trityl calibration compounds of the invention is that they may easily be produced to have almost any predetermined molecular weight. In particular, the trityl compounds may be produced by reacting a base reactant of formula (I) R-X with an amine. This produces an amide-substituted trityl compound. The reaction conditions may be chosen by those skilled in the art. Our earlier publication WO99/60007 describes suitable reaction conditions. In that publication we describe the use of trityl compounds as tag moieties for molecules such as oligonucleotides or oligopeptides. The techniques described there for modifying molecular mass of the trityl groups may be applied in the present invention.

According to a fourth aspect of the invention we provide a kit for the production of calibration compounds for mass spectrometry comprising:

- (a) at least one base reactant of formula (I) R-X where R is a trityl group and X is cleavable to form a charged species for mass spectrometry and
  - (b) at least two different amine compounds which are of different molecular masses and which are each capable of reacting with the base reactant
- and base reactant (a) is packaged separately from amine compounds (b).

This kit can be supplied to end users for reaction of base reactant (a) with amine compounds (b) as desired to create a group of compounds of formula (I) of different molecular masses. As in the earlier aspects of the invention, preferably the kit comprises at least 5, more preferably at least 10 and in some cases at least 20 different amine compounds which are of different molecular masses. Other preferred features discussed above may be applied as appropriate.

The kit can be supplied with instructions such that the end user selects at least two desired molecular masses  $M_1$  and  $M_2$  for the calibration compounds and chooses one or more amines for reaction with the base reactant so as to obtain compounds of formula (I) having the desired predetermined molecular masses  $M_1$  and  $M_2$ . These compounds may then be used in mass spectrometry.

In a fifth aspect of the invention we provide a further kit for the production of a set of calibration compounds comprising a first package comprising a base reactant of formula (I) R-X, in which R is a trityl group and X is cleavable to form a charged species for mass spectrometry, and at least two separate second packages (a) and (b), each containing a mixture of at least two amine compounds which have different molecular masses and which are capable of reacting with the base reactant.

Thus the kit is suitable for producing a set of calibration compounds according to the third aspect of the invention. As in that aspect, preferably the lowest molecular mass in mixture (a) is lower than the lowest molecular mass in mixture (b) and the highest molecular mass in mixture (a) is lower than the highest molecular mass in mixture (b). The kit may contain at least three, preferably at least five mixtures, so as to provide a greater number of mixtures. Preferably each mixture contains at least five different amine compounds, more preferably at least ten different amine compounds and in some cases at least twenty different amine compounds.

The invention will now be illustrated with reference to the following example.

Example

In this example the exact mass of a compound of formula  $C_{25}H_{20}NO_5$  is determined. The calculated exact mass is 414.13415. Tr(NHS) is produced as described by Shchepinov et al in Tetrahedron 56 (2000) 2713-2724. This compound is treated with butylamine and amylamine as described in that publication. This gives two calibration compounds with exact calculated masses of 402.50781 and 416.52096. The two calibration compounds and analyte are subjected to mass spectrometry and the monoisotopic mass of the analyte was determined to be 414.13621, with an error of 4.9ppm.

CLAIMS

1. A method of measuring the molecular mass of a compound Y of unknown molecular mass by mass spectrometry,  
5 comprising  
    providing a sample of compound Y,  
    providing samples of at least two different compounds each of formula (I), R-X in which R is a trityl group and X is cleavable to form a charged species for mass  
10 spectrometry,  
    and recording the molecular mass of compound Y and the at least two compounds of formula (I) in a mass spectrometer.
2. A method according to claim 1 in which R is  $R^1R^2R^3C$ - wherein  $R^1$ ,  $R^2$  and  $R^3$  are the same or different and each is  
15 a monocyclic or fused ring aromatic group - that is substituted or unsubstituted.
3. A method according to claim 2 in which at least one of  $R^1$ ,  $R^2$  and  $R^3$  carries a substituent selected from  $C_1$ - $C_{20}$  alkoxy or hydrocarbyl, substituted or unsubstituted.
- 20 4. A method according to claim 3 in which the alkoxy or hydrocarbyl is substituted by carboxylic acid, sulphonic acid, nitro, cyano, hydroxyl, thiol, primary, secondary or tertiary amino, primary or secondary amido, anhydride, carbonyl halide or active ester.
- 25 5. A method according to any of claims 2 to 4 in which each of  $R^1$ ,  $R^2$  and  $R^3$  is aryl, preferably phenyl.
6. A method according to any preceding claim in which the trityl group R includes two or four or more amide substituents.
- 30 7. A method according to any of claims 2 to 6 in which  $R^1$ ,  $R^2$  and  $R^3$  together carry at least two amide groups and/or at least two reactive groups for coupling, preferably N-hydroxysuccinimide ester groups.
8. A method according to any preceding claim in which X  
35 is halide or tosylate.
9. A method according to any preceding claim comprising providing at least five, preferably at least ten compounds

of formula (I).and recording their molecular masses in a mass spectrometer.

10. A method according to any preceding claim in which the group X is photocleavable to form a charged species for mass spectrometry.

11. A method according to any preceding claim additionally comprising estimating the molecular mass of unknown compound Y as  $M_y$  and providing at least one compound of formula (I) which has known molecular mass  $M_1$  below  $M_y$  and at least one different compound of formula (I) which has molecular mass  $M_2$  above  $M_y$ , and preferably the difference between  $M_y$  and each of  $M_1$  and  $M_2$  is not more than  $\pm 50\%$

12. A method according to any preceding claim additionally comprising providing a sample of at least one further compound Z of unknown molecular mass and measuring the molecular mass of compound Z.

13. Use of a compound of formula (I) R-X in which R is a trityl group and X is cleavable to form a charged species for mass spectrometry as a calibration compound for mass spectrometry.

14. A kit for the production of calibration compounds for mass spectrometry comprising:

(a) at least one base reactant of formula (I) R-X where R is a trityl group and X is cleavable to form a charged species for mass spectrometry and

(b) at least two different amine compounds which are of different molecular masses and which are each capable of reacting with the base reactant

and base reactant (a) is packaged separately from amine compounds (b).

15. A kit according to claim 14, additionally comprising instructions to select at least two desired molecular masses  $M_1$  and  $M_2$  for the calibration compounds and to choose one or more amines for reaction with the base reactant so as to obtain compounds of the desired predetermined molecular masses  $M_1$  and  $M_2$ , and instructions to use the compounds in mass spectrometry.

16. A set of calibration compounds for mass spectrometry comprising at least two separately packaged mixtures (a) and (b), wherein

5 mixture (a) comprises at least two different compounds each of formula (I) R-X and having different molecular masses

10 mixture (b) comprises at least two further compounds of formula (I) R-X having different molecular masses and wherein R is a trityl group and X is cleavable to form a charged species for mass spectrometry.

17. A set according to claim 16 in which the lowest molecular mass in mixture (a) is lower than the lowest molecular mass in mixture (b) and the highest molecular mass in mixture (a) is lower than the highest molecular mass in mixture (b).

18. A set according to claim 16 or claim 17 in which each of mixtures (a) and (b) contains at least five different compounds of different molecular masses, preferably at least 10 different compounds of different molecular masses.

20 19. A set according to any of claims 16 to 18 comprising at least three separately packaged mixtures of compounds, preferably at least five separately packaged mixtures of compounds.

25 20. A kit for the production of a set of calibration compounds comprising a first package comprising a base reactant of formula (I) R-X, in which R is a trityl group and X is cleavable to form a charged species for mass spectrometry, and at least two separate second packages (a) and (b), each containing a mixture of at least two amine compounds which have different molecular masses and which are capable of reacting with the base reactant.

30 21. A kit according to claim 20 in which the lowest molecular mass in mixture (a) is lower than the lowest molecular mass in mixture (b) and the highest molecular mass in mixture (a) is lower than the highest molecular mass in mixture (b).



22. A kit according to claim 20 or claim 21 in which each of mixtures (a) and (b) contain at least five different amine compounds of different molecular masses, preferably at least ten different amine compounds of different molecular masses.

23. A kit according to any of claims 20 to 22 comprising at least three mixtures of amine compounds, preferably at least five mixtures of amine compounds.

24. A method of measuring the molecular mass of a compound Y of unknown molecular mass comprising estimating the expected molecular mass of compound Y, selecting at least one calibration compound of formula (I) R-X having molecular weight close to the expected molecular weight of the compound Y, in which R is a trityl group and X is cleavable to form a charged species for mass spectrometry and subjecting both compounds to mass spectrometry simultaneously.

25. A method according to claim 24 in which the calibration compound R-X is provided by selecting a base reactant R-X in which R is a trityl group different from R and selecting an amine reactant of appropriate molecular mass and reacting the amine reactant and the base reactant.

26. A mixture of at least two compounds of formula (I) R-X in which R is a trityl group and X is a group cleavable to give a charged species for analysis by mass spectrometry.

27. A mixture according to claim 26 comprising at least five, preferably at least ten different compounds of formula (I).

28. A method of mass spectrometry comprising subjecting simultaneously to mass spectrometry at least two different compounds of formula (I) R-X in which R is a trityl group and X is cleavable to give a charged species for analysis by mass spectrometry.

